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PRINCIPAL INVESTIGATOR: Bouchra Edderkaoui., Ph.D.

CONTRACTING ORGANIZATION: Loma Linda Veterans Association for Research and Education
Redlands, CA 92373

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Bouchra Edderkaoui, Ph.D.

E-Mail: Bouchra.edderkaoui@va.gov

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Loma Linda Veterans
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710 Brookside Ave. STE 2
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13. SUPPLEMENTARY NOTES

Injuries causing ruptures to the anterior cruciate ligament (ACL) are a common cause of posttraumatic osteoarthritis (PTOA)

Since Duffy antigen receptor for chemokines (DARC) binds inflammatory chemokines previously shown to be up-regulated in osteoarthritic knees, we proposed to analyze the role of DARC on the development of PTOA in the present study.

The mRNA levels of two major inflammatory cytokines *Il-6*, *Il-1β* were up-regulated as early as at one-day post-ACL injury. While no effect was observed in the mRNA level of *Rantes* in response to ACL injury. *Mcp-1* mRNA was increased at one-day post-ACL injury in Darc-KO and wild type mice but the magnitude of increase was greater in Darc-KO compared to WT mice at one-day post-ACL injury. Among the three *Mmps* tested in this study, only *Mmp3* showed significant increase in mRNA level in response to ACL injury during the first week post-ACL injury in both lines of mice.

At 8-weeks post-ACL injury, injured knees showed a significant loss of articular cartilage, that reached calcified cartilage layer with no significant difference between Darc-KO and WT mice suggesting that lack of DARC expression in the whole body does not affect PTOA development. We still need to analyze more histologic sections from knees collected at different time points, to better understand the relationship between the development of PTOA pathology and the changes in gene expression we have observed, and we need to determine if local blockade of DARC function at the knee joint have a different effect on PTOA development than the whole-body loss of DARC function.

15. SUBJECT TERMS
DARC, ACL, Inflammatory, PTOA

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A. INTRODUCTION

The anterior cruciate ligament (ACL) connects the femur to the tibia at the knee joint, and is critical in providing stability of the knee. Injuries causing ruptures to the ACL are a common cause of knee instability and subsequent posttraumatic osteoarthritis (PTOA). US military personnel suffer from ACL injury at a rate 10 times that of the civilian population. In addition, even though ACL injuries can be surgically repaired, following reconstructive ACL surgery, 35% of patients will develop tibio-femoral osteoarthritis (OA) (Svoboda, 2014). Clearly, ligament damage, especially to the ACL is a significant factor in the initiation and progression of PTOA, and merits further study in order to develop effective therapeutic interventions.

Hypotheses. Based on the above findings, we proposed the following hypotheses: (**1**) Anterior cruciate ligament (ACL) injury/rupture induces cytokine secretion in the synovial space, leading to increased chemokine expression; (**2**) then, through DARC-dependent chemokine transcytosis will increase chemokine secretion, in turn promoting the recruitment of inflammatory cells to the joint space, which will lead to cartilage degradation and PTOA; and (**3**) the local application of anti-DARC antibody to the joint space of the knee after ACL injury will inhibit chemokine binding to DARC, reducing the recruitment of inflammatory cells to the joint space, and reducing or preventing the onset and/or progression of PTOA.

Objectives. Developing treatments for PTOA requires an understanding of the underlying molecular mechanisms. For this purpose, we proposed to first identify those chemokines that bind to DARC, and whose expression is induced in response to ACL injury. Time course experiments will identify the temporal relationship between DARC-chemokine interactions and the development of PTOA pathology. Together, these studies will provide critical information about the molecular mechanisms that contribute to inflammation and cartilage damage, and will identify when chemokine-DARC interaction occurs. This will aid in the design of the second series of experiments to evaluate, in a mouse model of PTOA that mimics joint injury in humans, the effect of local administration of DARC neutralizing antibodies on PTOA development, as well as potentially identifying other targets that may be amenable for therapeutic intervention.

Therefore, we proposed two specific aims to confirm the above hypothesis:

Specific Aim 1. Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA

Specific Aim 2. Develop a strategy to locally reduce or inhibit post-ACL injury mediated inflammation and development of PTOA by direct administration of anti-DARC antibodies to the injured knee

B. BODY

Progress report during the first year of the funding period

1. **Specific Aim 1.** Identify the chemokines induced and the cells that migrated to the synovial fluid in response to knee injury during the development of PTOA

   a. **Gene expression profile in response to ACL injury caused by axial loading**

   - **Animal Model.** Optimize the conditions for axial loading to induce ACL tear/rupture. 10-week old C57/B6 wild type (WT) and Darc-KO mice were used during this funding period.

Mice were anesthetized by isoflurane inhalation. The right leg was subjected to a preload of 1-2N applied to the knee, followed by the single dynamic axial compressive load (at a 40 N/s loading rate), of 12 N as previously described (**Christiansen et al., 2012**). This force was chosen based on previous studies showing 12N was a threshold force in B6 mice where ACL rupture would occur randomly (**Christiansen et al., 2012**). However, in our hands using our Instron Hydraulic machine, the 12N targeted force did not induce any obvious injury to the knee, so we increased the load to 15 N, which was successful to induce knee injury, characterized by a discontinuity in the force-displacement curve at 12N (**Fig. 1**). Subcutaneous injection of buprenorphine analgesia (0.5 μg/g body weight) was administered to each animal after injury. The animals were allowed unrestricted movement following injury. The left knee served as contralateral control.

Animals were sacrificed at different time points post-ACL injury. Tissues were collected by excising a region extending 1-2 mm above and below the middle of the knee joint, with samples snap frozen in liquid nitrogen, and stored at -80°C. For RNA extraction, samples were pulverized in liquid nitrogen; total RNA was isolated using Trizol and RNeasy kit (Qiagen) and processed for real-time-PCR. Real-time quantitative PCR was performed using the Applied Biosystems ViiA7 RT-PCR systems instrument, and the SYBR Green PCR kit from Applied Biosystems Inc.

- **Gene expression profile post-axial loading**

  - <u>Gene expression profile at one-day post-ACL injury</u>

The expression levels of the two major inflammatory cytokines; *Il-6* and *Il-1beta*(*β*) as well as *CD14;* the marker of monocytes, were evaluated at one-day post-knee injury, using Real Time PCR as previously described (Edderkaoui et al., 2007).

In samples collected from knees from WT and Darc-KO mice, we observed a 7- 8-fold increase in the mRNA level of *Il-6* at one-day post-knee injury (**Fig. 2**). In addition, the mRNA levels of Il-1β and CD14 were similarly increased at one-day post knee injury in both lines of mice (**Fig. 2**).

DARC binds to several chemokines previously shown to be present in OA knees (e.g., IL-8, CXCL1, MCP-1/CCL2 and RANTES/CCL5) (Bay-Jensen et al., 2015), and which are known to be involved in cell migration. Thus, to determine the role of these chemokines in PTOA development, we first evaluated the change in their mRNA levels in response to knee injury. We predicted that expression of these chemokines would correlate with the level of inflammation in the knee, and the magnitude of catabolic effects on cartilage and subchondral bone that occur in response to injury.

At one day-post knee injury, no significant increase was observed in the mRNA level of *Rantes* in any mouse line. However, in experimentally injured knees collected from Darc-KO mice we observed an increase in mRNA levels of *Mcp-1* as compared to WT mice. This could in part be due to the difference in basal levels of Mcp-1 mRNA, which were higher in Darc-KO mice as compared to WT mice.

Since it has been reported (Loeser et al., 2012) that level of CCL21 was found increased in osteoarthritic knees, the mRNA level of Ccl21 was evaluated at one-day post-knee injury. While a small reduction in the expression of CCl21 was found in the injured knees collected from WT mice as compared to non-injured knees, a significant reduction in the mRNA level of CCl21 was observed in the injured knees collected form Darc-KO mice at one-day post-knee injury (**Fig. 3**).

The mRNA levels of three matrix metalloproteinases known for their catabolic effects on the extracellular matrix, MMP3, MMP9 and 13, were evaluated at one-day post-knee injury. Only *Mmp3* showed significant increase in mRNA levels when compared to unloaded knees at one-day post knee injury in both lines of mice (**Fig. 4**). However, the magnitude of increase in the mRNA level was less in Darc-KO mice than was observed in WT mice (**Fig. 4**). Again, it is possible that this could be due to lower basal levels of Mmp3 mRNA in Darc-KO compared to the WT mice.

- Gene expression profile at three days post-axial loading

At three days post axial-loading and ACL injury, among the three chemokines tested, only Ccl21 mRNA level was significantly increased in the injured knee compared to the un-injured knee in both lines of mice (**Fig. 5**). Mcp-1 and Rantes mRNA levels were not significantly different in injured and un-injured knees, but Mmp3 mRNA levels remained higher in the injured knees compared to un-injured knees in both WT and Darc-KO mice (**Fig. 6**), suggesting a relationship between CCL21 and MMP3 expression.

b. Histologic assessment of knee joint after meniscectomy

Injured and un-injured knee joints were collected and muscle tissue was removed. The knees were fixed for one day in 10% buffered formalin, decalcified in Formical 4 for one day at room temperature and embedded in paraffin. Then, 5 µm sections were taken at 100-µm intervals from the posterior to anterior side of the knee joints. Slides were stained with Safranin-O/Fast green to assess general morphology and matrix proteoglycans.

At 3 days post knee injury, the knee joints showed infiltration of several inflammatory cells both at the connection ACL – femoral condyle and at to the synovium (**Fig. 7**).

Specimens from injured knees showed a significant loss of articular cartilage, not only at the superficial zone but also in the calcified cartilage layer at 8-weeks post injury. There was no significant difference in cartilage loss between the two lines of mice at this time point (Fig. 8B). In contrast, in the sham un-injured knees, the superficial layer of cartilage was smooth and no disruption of surface integrity was observed. The cartilage matrix was well stained with Safranin-O. Preservation of Safranin-O staining and chondrocytes in calcified cartilage layer and smooth bony trabeculum was observed in subchondral layer (**Fig. 8A**).

C. KEY RESEARCH ACCOMPLISHMENTS DURING THE LAST 12 MONTHS OF FUNDING

We have made the following progress towards achieving the specific aims in this research project:

- We have optimized the conditions of the axial loading model to induce ACL injury
- We have evaluated the expression of the two major pro-inflammatory cytokines and the expression of monocyte marker CD14 at one-day post-knee injury.
- We observed a significant increase in the expression of *Il-6*, Il-1β as well as CD14 at one-day post-knee injury in both lines of mice; no difference in the expression was observed between the two lines of mice in response to knee ACL injury.
- We have evaluated the change in the expression of the two chemokines that bind to DARC and the expression of *Ccl21* as well as the two major effectors of the metabolism of articular cartilage, *Mmp3* and *Mmp13*at one and three days post knee injury.
- The greatest change in gene expression was found in the expression of Mmp3 as early as one-day post knee injury.
- *Ccl21* mRNA level was induced later on in the injured knees and became significantly greater compared to un-injured knees, at three days post knee injury in both lines of mice.
- Our examination of the sections prepared from knees collected at 3 days post-knee injury revealed inflammatory secretions and the presence of abundant mononuclear cells in the synovium.
- Our evaluation of the sections prepared from knees collected at 8 weeks post-knee injury revealed the presence of chondrocyte loss, and a loss of Safranin staining, which extends into the radial zone. While the sections derived from the sham operated knees showed smooth superficial layer of cartilage, no disruption of surface integrity was observed. The cartilage matrix was well stained with Safranin-O. Preservation of Safranin-O and chondrocytes in calcified cartilage layer and smooth, bony trabeculum was observed in subchondral layer.

D. CONCLUSION

Knee ACL injury caused by axial loading induces the expression of multiple genes at different time points. Expression of Il-6 and Il-1β, the major pro-inflammatory cytokines, and Mmp3, known for its role in cartilage degradation, was induced as early as one day post-knee injury, but expression of the Ccl21 chemokine was induced later at the injured knees, with significantly increased mRNA level evident at three days post-knee injury. Furthermore, *Mmp3* mRNA levels remained significantly elevated in the injured knees compared to control knees during the first week post-knee injury in both lines of mice. Interestingly, no difference was observed in the response of the pro-inflammatory cytokines and chemokines between WT mice and Darc-KO mice suggesting that DARC is not involved in the inflammation induced by knee injury. Furthermore, no significant difference was observed in cartilage loss between the two lines of mice at 8-weeks post-knee injury. We are in the process of analyzing additional histologic sections prepared from knees collected at different time points to better understand the relationship

between the development of PTOA pathology and the changes in gene expression we have observed.

E. INVENTIONS, PATENTS AND LICENSES: Nothing to report

F. REPORTABLE OUTCOMES:

ACL injury induced the expression of Il-6, Il-1β, CD14 and Mmp3 as early as one day post injury. CCL21 expression was induced later with mRNA levels significantly higher in injured knees compared un-injured knees at three days post knee injury. At 8-weeks post knee injury, a great loss of articular cartilage, which extends into the radial zone was observed in both lines of mice.

G. OTHER ACHIEVEMENTS: Nothing to report

H. REFERENCES:

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I. FIGURE LEGENDS

Figure 1. ACL injury during tibial compression loading identified by a release of compressive force during the loading cycle, with a continued increase in actuator displacement. X axis represents the time in milliseconds, the Y axis represents the loading force in Newton.

Figure 2. mRNA expression levels of the two major inflammatory genes; *Il6* and *Il-1β* and monocyte marker *Cd14* at the knee joints at one day post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left knees. Rk., for right knee, and Lk., for left knee.

Figure 3. mRNA expression levels of the two inflammatory chemokines that bind to DARC and *Ccl21* at one day post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. $P<0.05 comparing the basal expression between the two lines of mice at the left knee. Rk., for right knee, and Lk., for left knee.

Figure 4. mRNA expression levels of the three major matrix metalloproteinases, *Mmp-3*, *Mmp-9* and *Mmp-13* at the knee joints , at one day post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left unloaded knees. #P<0.05 comparing the expression at the right knees between the two lines of mice. Rk., for right knee, and Lk., for left knee.

Figure 5. mRNA expression levels of the two inflammatory chemokines that bind to DARC and *Ccl21* at three days post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left unloaded knees. Rk., for right knee, and Lk., for left knee.

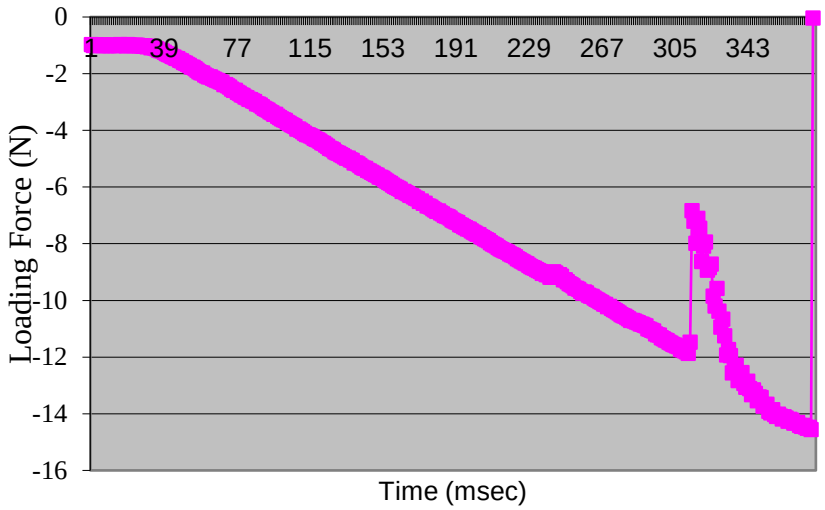
Figure 6. mRNA expression levels of the two major matrix metalloproteinases, *Mmp-3* and *Mmp-13* at the knee joints , at three- day post-ACL injury. We collected knees from both sham unloaded knees (left knees) and loaded knees (right knees), data are presented as fold change compared to WT unloaded knees, n=4 and **P*<0.05 vs WT left unloaded knees. Rk., for right knee, and Lk., for left knee.

Figure 7. Representative images from histologic sections of intact knee (A) and injured (B) knees at three days-post ACL injury. A. 1. Shows intact medial meniscus. **B.1.** Shows injured medial meniscus with inflammatory cells (red arrow) **A. 2.** Shows intact ligaments with no inflammation. **B.2.** Shows ruptured ACL with abundant inflammatory cells (red arrow). **A.3**. and **B.3.** represent the collateral sides of the un-injured knee and the injured knee, respectively. 1, 2 and 3 squares represent the areas that were 4x amplified.

Figure 8. Representative sections of the left intact knee and the right injured knees from WT and Darc-KO mice at 8-week post ACL injury. Intact knee showing smooth, with no disruption of the articular surface integrity. Injured knees from WT and Darc-KO mice showing irregularities, and severe loss of Safranin-O staining at the articular cartilage (blue arrows), mostly at the medial

side, with bone erosion. Sections were stained with Safranin-O/Fast Green and observed using microscope objective 1.25 x. t. for tibia plate and f. for femur condyle.

Fig. 1



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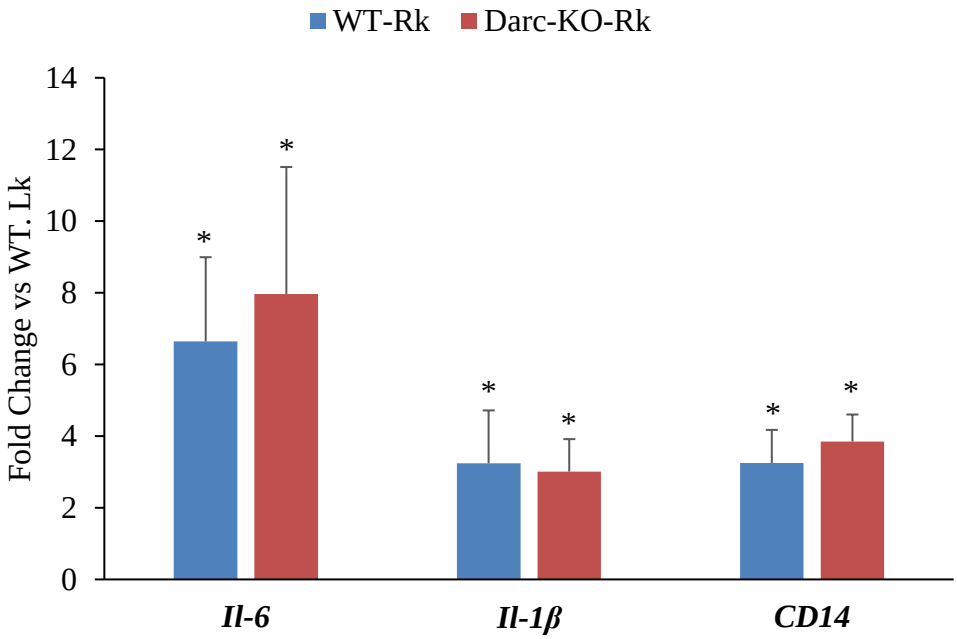

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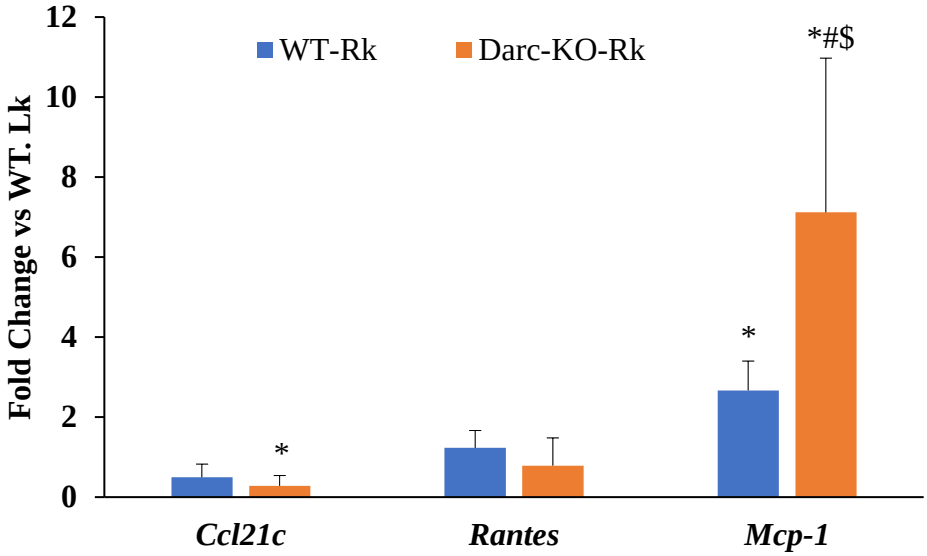
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Fig. 4

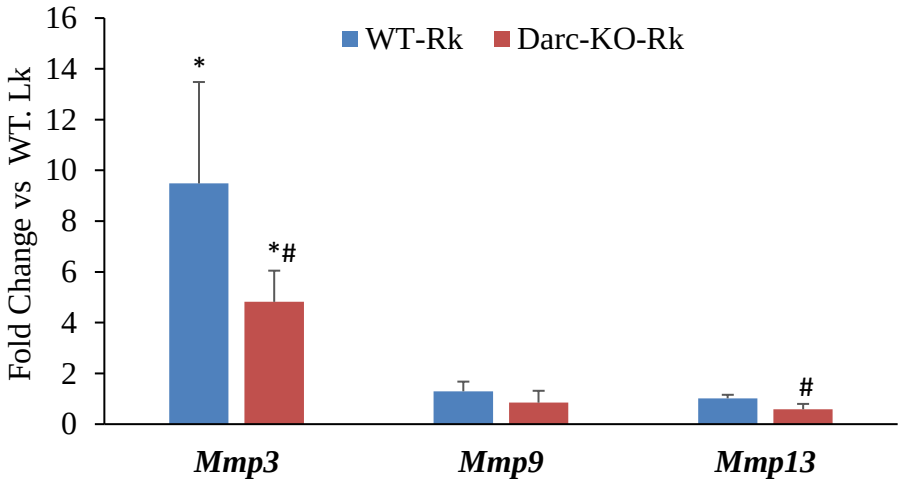
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Fig. 5

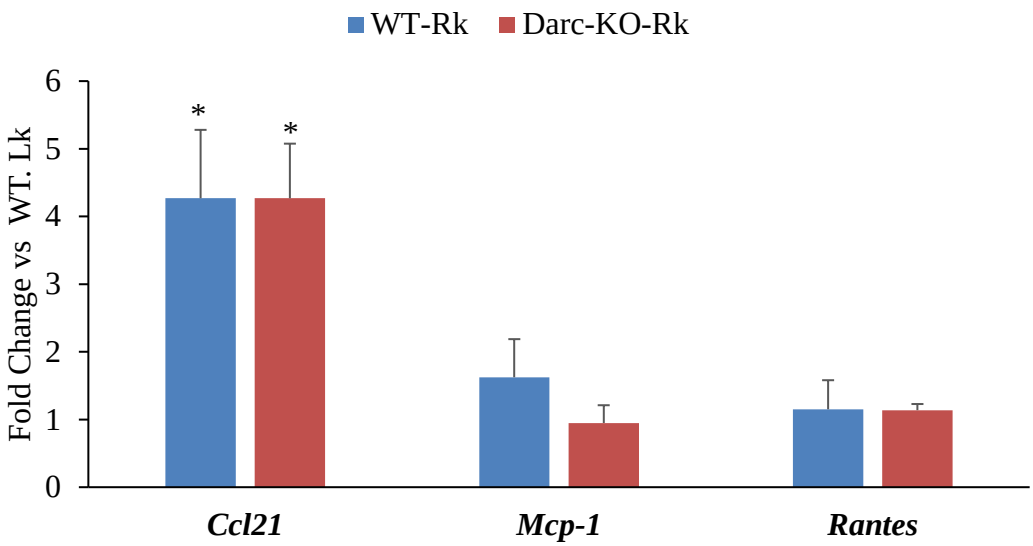
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Fig. 6

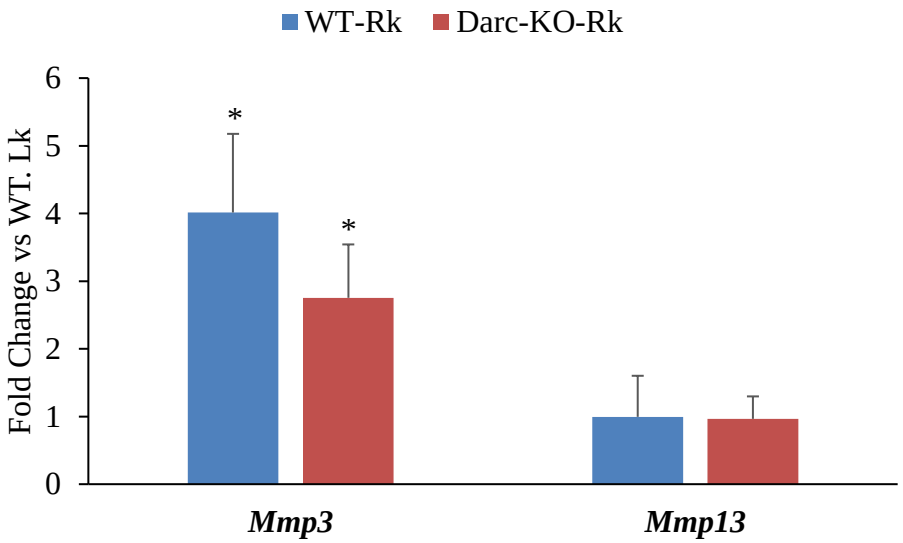
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Fig. 7

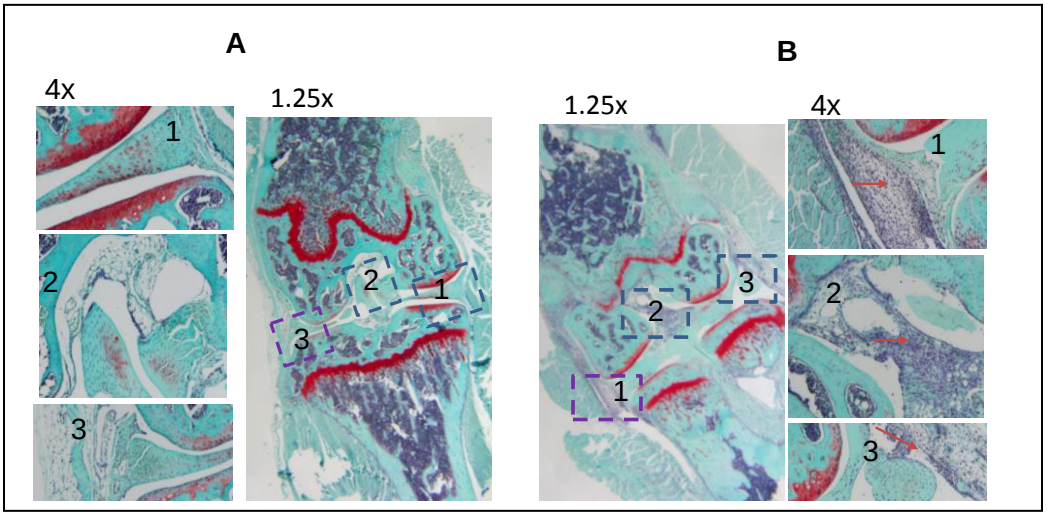
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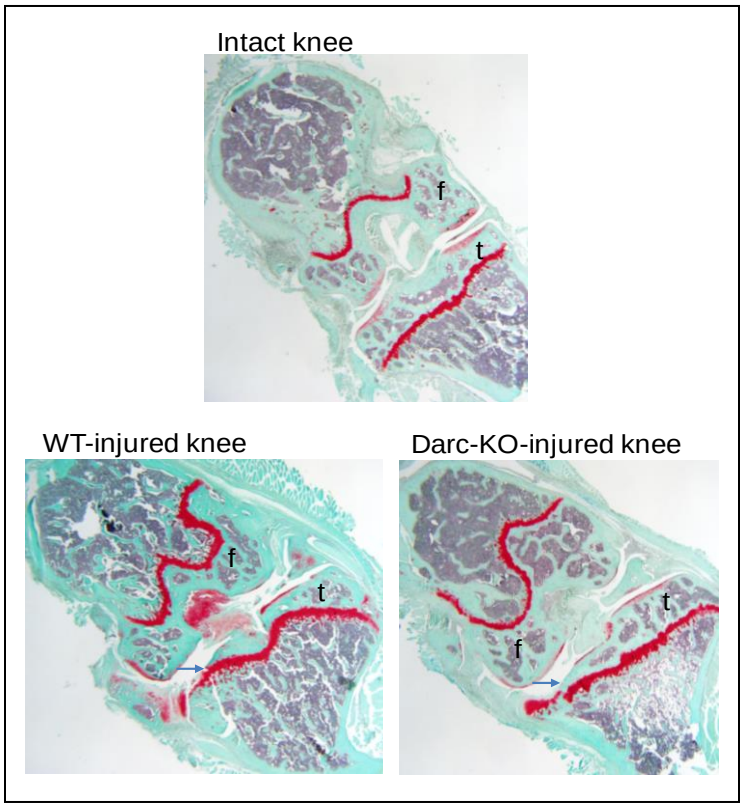
Fig. 8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